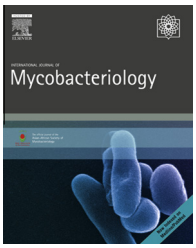
International Journal of Mycobacteriology 5 (2016) 280–287

Available at www.sciencedirect.com

ScienceDirect

journal homepage: www.elsevier.com/locate/IJMYCO



Full Length Article

Tuberculosis in Sardinia: An investigation into the relationship between natives and immigrants

Melania Ruggeri *, *Paola Molicotti*, *Marina Cubeddu*, *Sara Cannas*, *Alessandra Bua*, *Stefania Zanetti*

Department of Biomedical Sciences, University of Sassari, Sassari, Sardinia, Italy

ARTICLE INFO

Article history:
Received 29 April 2016
Received in revised form
31 May 2016
Accepted 1 June 2016
Available online 27 June 2016

Keywords:
Immigrants
Phylogenetic analysis
Tuberculosis
VNTR-MIRU genotyping

ABSTRACT

Objective/background: Tuberculosis (TB) has had a recrudescence in the last few decades in Italy as a result of many factors, among which migration from countries where TB is endemic is one of them. In Sardinia, a major island of Italy, there was no knowledge of the mechanisms of transmission of TB in the immigrant subpopulation and the impact it may have on the native subpopulation and on the community as a whole. Therefore, a molecular epidemiological study was carried out to get a clearer picture of the number and genetic features of *Mycobacterium tuberculosis* strains isolated from immigrants and from natives in Sardinia. *Methods:* Two groups of clinical isolates of *M. tuberculosis*, one collected from immigrants and the other one from Sardinians, were analyzed in this study. The genotyping was executed through the variable number tandem repeat-mycobacterial interspersed repetitive units technique and a first-line antimycobacterial drug-susceptibility test was also carried out. *Results:* Thirty-six clinical isolates from immigrants and 25 from Sardinians were analyzed. Variable number tandem repeat-mycobacterial interspersed repetitive units technique showed that all of them belonged to different strains and there was a quite high allelic diversity among them. Moreover, data collected allowed the finding of, with a good approximation, the phylogenetic relations among the strains isolated and the best-known phylogenetic groups. *Conclusion:* The study pointed out that since every strain is different, there was no TB transmission in any of the subpopulations and between immigrants and natives. This showed that the presence of immigrants was not a risk factor for contracting TB in the community.

* Corresponding author at: Department of Biomedical Sciences, University of Sassari, Viale San Pietro 43 B, Sassari, Sardinia 07100, Italy.
E-mail address: melaruggeri@libero.it (M. Ruggeri).

Peer review under responsibility of Asian African Society for Mycobacteriology.
http://dx.doi.org/10.1016/j.ijmyco.2016.06.002

Introduction

Tuberculosis (TB) is the leading cause of adult death due to a single infectious agent worldwide [1]. The most recent data of the annual report "Global Tuberculosis Control" of the World Health Organization estimated that there were 9.6 million new cases and 1.5 million deaths in 2014. The most affected geographical areas are low-income developing countries in Africa (280 new cases/100,000 inhabitants) and Asia (210 new cases/100,000 inhabitants); percentages are decidedly lower in Europe (40 new cases/100,000 inhabitants) and Americas (30 new cases/100,000 inhabitants). In industrialized countries, after a significant decrease in TB incidence over the last century, there has been a recrudescence, due especially to the massive immigration from low-income and middle-income countries where the illness is endemic, to the appearance of multidrug resistant (MDR) strains and to the lack of prevention and control programs [1].

In Italy, the incidence of TB has registered a progressive increase in the last few decades; however, it has remained constant—under 10 new cases/100,000 inhabitants—therefore, Italy is considered a low incidence country. This increase has not involved all people in the same way, but it has affected some social classes, among which are immigrants. In 2008, the incidence rate was 3.8 cases/100,000 for people born in Italy and 50–60 cases/100,000 for people born abroad [2–4]. Foreign-born people coming from countries with a high incidence of TB are a population at high risk to become sick: (1) firstly, because they come from areas in which the disease is endemic and therefore is highly probable to contract the infection; and (2) secondly, their standard of living both in their country of origin and in Italy is not often compatible with the maintenance of good health. Consequently, the risk of developing TB is higher than the national average [4–7]. Some studies have shown that the presence of immigrants in low-incidence countries did not increase the risk for the native population of contracting the disease [8–10]. However, the same studies suggested that the situation may change depending on the degree of interaction between immigrants and natives, as well as among immigrants of different nationalities, which are related to social factors specific for each place [8–10].

Also, in Sardinia, one of the two major islands of Italy, the migration rate has progressively increased, and at the same time the percentage of immigrants among TB patients has also increased [3]. Sardinia is a low-density population island, with 68 inhabitants/km^2 and a percentage of resident immigrants of 2.5% [4]. They come from TB high-incidence countries, mainly from the African regions of Maghreb and Senegal, from East Asia, and from East Europe. Most of them are well integrated in the social fabric, where they work especially as care givers or shoppers, and they frequent the same places of natives (i.e., schools and supermarkets) [5]. In order to plan effective control programs, it is necessary to know the dynamics of transmission both in the population as a whole and in the subpopulations of which it is composed, especially those at high risk such as the immigrants [6,11]. To understand the mechanism of transmission of TB in the immigrants' subpopulation in Sardinia and the impact it may have on the native subpopulation and on the community as a whole, a molecular epidemiological study was carried out to get a clearer picture of the number and genetic features of *Mycobacterium tuberculosis* strains isolated from immigrants and from natives in Sardinia.

Materials and methods

M. tuberculosis strains

Two groups of clinical isolates of *M. tuberculosis* collected over a 10-year period were analyzed and compared in this study. The first group comprised of 36 isolates from immigrants coming from Africa, East Europe, and Asia. The second group was made up of 25 isolates from Sardinians coming from different districts of the land.

DNA extraction

Before starting the genotyping, every clinical isolate was inoculated in Middlebrook 7H9 added with ADC (Albumin Dextrose Catalase) Microbiol diagnostici (Z.I. Macchiareddu, Uta-Cagliari) until the log phase was reached. Also, the reference strain *H37Rv* was inoculated and used as a positive control in the following steps. DNA was then extracted and purified from each mycobacterial culture and used for the genotyping through the N-cetyl-N,N,N-trimethyl ammonium bromide procedure, following standard protocols [11].

Variable number tandem repeat-mycobacterial interspersed repetitive units genotyping

The isolates were genotyped by PCR amplification of a set of 15 loci MIRU with a discriminatory power among isolates of 96% of the total resolution, specific for epidemiological investigations (Table 1) [12,13]. PCR amplifications were performed using three different mixtures, characterized by different concentrations of magnesium. For each locus, the one that granted the best results was chosen. Mixes were prepared with 2 μL of 10× buffer, 4 μL of Betaine (Sigma-Aldrich Merck), 1.6 μL (mix 1) or 1.2 μL (mix 2) or 0.8 μL (mix 3) of $MgCl_2$, 0.2 μL of dATP, dCTP, dGTP and dTTP, 0.8 μL of each primer, 0.08 μL di DNA-polymerase and distilled water up to 18 μL. 2 μL of the mycobacterial DNA previously extracted and purified were added to the mix, for a final volume of 20 μL [14,15].

PCR were run in a thermal cycler, under the same condition for each locus: 95 °C for 15 min, 40 cycles of 94 °C for 1 min, 59 °C for 1 min, and 72 °C per 1:30 min, followed by 72 °C for 10 min. PCR products were analysed by 2% agarose gel electrophoresis, using 100-bp and 50-bp DNA ladders [14,15]. For each isolate and each locus, the number of the allelic repetitions was calculated through a comparison between the lenght of the amplicon obtained and the length of the amplicons of H37Rv.

In this way it was possible to associate to each isolate a numerical code formed by the allelic repetitions for each locus considered in the same order for all the isolates.

In the elaboration of the results, an index of allelic diversity D was calculated with the following formula:

Table 1 – Loci and primers used in in genotyping.

Locus	Repeat unit length (bp)	PCR primer pairs (5′–3′)
424	51	CTTGGCCGGCATCAAGCGCATTATT
		GGCAGCAGAGCCCGGGATTCTTC
577	58	CGAGAGTGGCAGTGGCGGTTATCT
		AATGACTTGAACGCGCAAATTGTGA
580	77	GCGCGAGAGCCCGAAATGC
		GCGCAGCAGAAACGCCAGC
802	54	GGGTTGCTGGATGACAACGTGT
		GGGTGATCTCGGCGAAATCAGATA
960	53	GTTCTTGACCAACTGCAGTCGTCC
		GCCACCTGGTGATCAGTCTACCT
1644	53	TCGGTGATCGGGTCCAGTCCAAGTA
		CCCGTCGTGCAGCCCTGGTAC
1955	57	AGATCCCAGTTGTCGTCGTC
		CAACATCGCCTGGTTCTGTA
2163	69	CGTAAGGGGGATGCGGGAAATAGG
		CGAAGTGAATGGTGGCAT
2165	75	AAATCGGTCCCATCACCTTCTTAT
		CGAAGCCTGGGGTGCCCGCGATTT
2401	58	CTTGAAGCCCCGGTCTCATCTGT
		ACTTGAACCCCCACGCCCATTAGTA
2996	51	TAGGTCTACCGTCGAAATCTGTGAC
		CATAGGCGACCAGGCGAATAG
3192	53	ACTGATTGGCTTCATACGGCTTTA
		GTGCCGACGTGGTCTTGAT
3690	58	CGGTGGAGGCGATGAACGTCTTC
		TAGAGCGGCACGGGGGAAAGCTTAG
4052	111	AACGCTCAGCTGTCGGAT
		CGGCCGTGCCGGCCAGGTCCTTCCCGAT
4156	59	TGACCACGGATTGCTCTAGT
		CGGCCGTGCCGGCCAGGTCCTTCCCGAT

PCR = polymerase chain reaction.

$D = 1 - 1/[n\ (n-1)]\ \sum x^2$, where x is the number of isolates with the same frequency at a given locus and n is the number of all the isolates.

Antimycobacterial susceptibility test

All the strains were tested for their susceptibility to isoniazide and rifampin. The tests were executed through two different methods; the first was based on the kit BACTEC MGIT 960 SIRE, with MGIT vials and the instrument BACTEC MGIT 960 (Becton, Dickinson and Company Franklin Lakes, New Jersey, US); the second is REMA (RESAZURINE MICROTITER ASSAY) TEST [16].

Results

In this study we analyzed and compared two groups of *M. tuberculosis* clinical isolates. The first group was composed of 36 isolates of immigrant patients of different ethnic groups and the second group was composed of 25 isolates of Sardinian patients coming from different districts of the island.

Regarding the first sample, 17 tubercular isolates were collected from East-European immigrants (15 from Romania and two from Russia), 14 from Africans (nine from Maghreb and five from Senegal), five from Asians (four from China and one from the Philippine Islands). Results reported in Tables 2 and 3 show that all the isolates were different from each other.

Allelic diversity for each locus, expressed by an index of allelic diversity D, was on the whole high, with an average $D = 0.75$. However, D for each locus was quite variable: the lowest value was registered for locus 580, for which $D = 0.44$, followed by loci 2163, loci 960, and loci 2165, for which D was respectively $D = 0.62$, $D = 0.66$, and $D = 0.67$; the highest value of D was found for loci 424, loci 802, and loci 4156, for which $D = 0.78$ (Table 3).

Grouping of the results on an ethnic basis did not show significant tendencies in the strains' distribution; allelic frequencies are distributed in a uniform way in the different loci, apart from some MIRU and some ethnic groups.

Table 2 shows that in locus 424 all the strains from Asian patients had five allelic repetitions; in locus 2996 all Romanian strains had six repetitions; in locus 4052 all Asian strains had eight repetitions and a frequency was also found in a strain from Maghreb (Table 2); in locus 1644 there was a higher variability in the Romanian group since some strains had five repeated copy numbers, a characteristic found only in this group.

The results of the antimycobacterial susceptibility test are shown in Table 4. Thirty-one of the 36 tested strains (86%) did not have any resistance. In the other five (14%), two were resistant only to isoniazid (5.5% of total strains) and one to rifampicin (2.7% of total strains), and two were MDR strains

Table 2 – Variable number tandem repeat-mycobacterial interspersed repetitive units (VNTR-MIRU) genotyping of immigrants' clinical isolates.

ID	Nationality	VNTR-MIRU loci														
		424	577	580	802	960	1,644	1,955	2,163	2,165	2,401	2,996	3,192	3,690	4,052	4,156
1	Chinese	0	5	3	3	3	0	5	3	0	4	8	4	2	8	1
2	Chinese	5	5	3	3	4	4	0	3	4	4	7	3	2	8	1
3	Chinese	3	5	2	3	3	4	6	0	4	0	7	4	0	8	0
4	Chinese	5	5	3	0	3	4	6	0	4	5	0	0	2	8	1
5	Maghrebian	3	5	2	1	5	0	0	2	3	4	2	3	0	8	3
6	Maghrebian	0	7	0	3	6	2	3	0	0	4	4	4	3	6	0
7	Maghrebian	1	4	3	0	3	4	2	0	3	3	6	4	1	5	1
8	Maghrebian	3	0	4	0	0	2	3	0	3	3	6	0	1	0	0
9	Maghrebian	0	5	3	3	3	2	0	2	0	0	0	4	1	5	1
10	Maghrebian	4	5	3	3	3	0	2	2	2	0	4	3	0	6	1
11	Maghrebian	3	5	3	6	3	2	2	0	3	3	0	0	3	0	0
12	Maghrebian	3	5	4	6	4	4	3	2	0	2	6	0	1	8	4
13	Maghrebian	4	5	3	2	4	4	3	2	2	3	6	2	1	6	0
14	Senegalese	2	3	3	1	5	4	4	5	3	5	0	2	2	4	4
15	Senegalese	0	0	3	1	4	0	2	0	0	3	6	0	5	0	4
16	Senegalese	2	5	3	1	3	4	2	5	3	3	4	3	3	5	2
17	Senegalese	3	0	4	6	3	2	0	0	3	3	5	2	3	5	4
18	Senegalese	2	0	3	0	5	4	2	0	3	5	6	2	3	0	4
19	Romanian	2	0	3	2	0	0	0	0	0	2	6	0	0	6	0
20	Romanian	2	5	3	1	3	4	3	2	2	2	6	3	1	6	3
21	Romanian	0	0	3	3	3	5	2	4	2	0	6	3	2	0	2
22	Romanian	2	3	3	5	5	4	0	4	3	5	6	1	0	4	4
23	Romanian	0	3	4	2	0	0	0	0	0	3	6	0	0	6	0
24	Romanian	4	7	4	6	3	4	2	0	4	3	0	2	1	0	3
25	Romanian	3	3	3	2	3	5	0	0	2	3	6	2	2	4	3
26	Romanian	3	3	3	2	3	5	2	3	2	3	6	3	2	4	3
27	Romanian	0	0	3	1	5	1	2	3	2	3	6	3	1	0	2
28	Romanian	2	4	0	2	5	4	4	0	2	0	5	3	2	4	3
29	Romanian	3	5	3	2	3	1	2	2	2	3	6	3	3	1	2
30	Romanian	3	0	3	0	5	2	0	0	2	0	6	3	2	0	4
31	Romanian	4	4	3	1	5	2	4	0	2	3	6	2	1	4	4
32	Romanian	0	4	3	3	5	2	0	0	4	3	6	0	1	6	4
33	Romanian	4	7	3	0	3	1	2	0	2	3	0	3	4	0	0
34	Russian	4	0	0	3	0	2	0	0	0	0	0	3	0	0	4
35	Russian	4	5	3	4	3	2	3	7	4	4	6	5	2	0	2
36	Philippine	5	5	3	0	4	4	2	0	2	3	0	3	2	8	2

ID = identification.

(5.5% of the total strains). Regarding the distribution of antimycobacterial resistance in each ethnic group, among people coming from Maghreb there were two resistant strains, one with one resistance to isoniazid and one MDR; among the Romanians there was one strain resistant to isoniazid and one to rifampicin; among the Russians there was one MDR strain; the strains isolated from immigrants from the other countries had no resistances.

Regarding the sample of isolates from Sardinian patients, they were collected in different districts: 14 from Sassari, eight from Nuoro, one from Tempio, one from Olbia, and one from Oristano.

Results, expressed in Tables 5 and 6, show that in this group the isolates were all different from each other, and there were no differences related to the place of origin.

The allelic diversity for each locus, expressed by the allelic diversity index *D*, was on the whole medium–high, with an average $D = 0.65$. For each locus it was quite variable. The lowest value was registered for locus 580, in which $D = 0.31$, followed by loci 2996 and 1644 with values $D = 0.37$ and $D = 0.43$, respectively. The highest values for *D* were for loci 802 and 4052, where $D = 0.82$ and $D = 0.81$ (Table 6). The low variability observed for locus 580 was due to the fact that almost all the strains analyzed had three repeated copies, for locus 2996 it was due to the fact that most strains had six allelic repetitions, while locus 1644 had two (Tables 5 and 6).

The results of the antimycobacterial susceptibility test are shown in Table 7. Twenty-one of the 25 tested strains (84%) did not have any resistance. Regarding the other four (16%), they were resistant to isoniazid and there were no MDR strains.

A comparison between the two populations analyzed (Tables 2, 3, 5 and 6) showed that there were no identical strains; moreover, the allelic diversity was lower in the Sardinian population ($D = 0.65$ vs. $D = 0.75$). In both groups, locus 580 showed the lowest genetic variability, since 77% of the strains analyzed had three allelic repetitions; in the remaining strains the allelic repetitions were two or four.

Table 3 – Allelic diversity index (*D*) for loci of immigrants' strains.

MIRU	Allelic frequencies								*D*
	1	2	3	4	5	6	7	8	
424	1	7	10	7	3	0	0	0	*D* = 0.78
577	0	0	4	4	16	0	7	0	*D* = 0.70
580	0	2	27	5	0	0	0	0	*D* = 0.44
802	7	6	9	2	1	4	0	0	*D* = 0.78
960	0	0	17	5	10	1	0	0	*D* = 0.66
1644	3	11	3	14	5	0	0	0	*D* = 0.72
1955	0	12	6	3	1	2	0	0	*D* = 0.72
2163	0	7	4	2	0	1	0	0	*D* = 0.62
2165	0	13	9	4	0	0	0	0	*D* = 0.72
2401	0	3	17	5	6	0	0	0	*D* = 0.72
2996	0	1	0	3	2	19	2	1	*D* = 0.67
3192	1	8	13	5	1	0	0	0	*D* = 0.75
3690	10	11	6	1	1	0	0	0	*D* = 0.73
4052	1	0	0	7	4	7	0	9	*D* = 0.76
4156	6	6	6	10	0	0	0	0	*D* = 0.78
									Average = 0.75

MIRU = mycobacterial interspersed repetitive units.

Table 4 – Antimycobacterial susceptibility pattern of immigrants' strains.

ID	Nationality	INH	RIF
1	Chinese	S	S
2	Chinese	S	S
3	Chinese	S	S
4	Chinese	S	S
5	Maghrebian	S	S
6	Maghrebian	S	S
7	Maghrebian	S	S
8	Maghrebian	S	S
9	Maghrebian	S	S
10	Maghrebian	R	S
11	Maghrebian	R	R
12	Maghrebian	S	S
13	Maghrebian	S	S
14	Senegalese	S	S
15	Senegalese	S	S
16	Senegalese	S	S
17	Senegalese	S	S
18	Senegalese	S	S
19	Romanian	S	S
20	Romanian	S	S
21	Romanian	S	S
22	Romanian	S	S
23	Romanian	S	S
24	Romanian	S	R
25	Romanian	S	S
26	Romanian	R	S
27	Romanian	S	S
28	Romanian	S	S
29	Romanian	S	S
30	Romanian	S	S
31	Romanian	S	S
32	Romanian	S	S
33	Romanian	S	S
34	Russian	S	S
35	Russian	R	R
36	Philippine	S	S

INH = isoniazid; R = resistant; RIF = rifampin; S = susceptible.

The other loci of the immigrant strains were characterized by a higher variability: the second locus with the lowest value was 2163, with $D = 0.62$, a little lower than the average value for all loci found in the native population. In the Sardinian population the second locus with the lowest value was 2996, with $D = 0.37$. In the immigrants the same locus had $D = 0.67$, which was significantly higher. This difference was due to the fact that most strains in both populations had six repetitions, but their number was proportionately higher in the native population (76% vs. 52.7%). In Sardinian there was also another MIRU for which the allelic variability was medium–low, while in immigrants it was very high ($D = 0.43$ vs. 0.72); in the first, 72% of the strains had two repetitions, in the second, two were the most found frequencies: four, in 39% of the strains, and two, in 30%; there were no Sardinian strains with four allelic repetitions.

As regards loci with the greatest allelic diversity, MIRU 802 in both populations showed the highest values for *D*; the other loci were different, but for the same MIRU the values for *D* were comparable, since they were included in the range 78–82. The distribution of the frequencies was also comparable for each locus.

According to results from the antimycobacterial susceptibility test (Tables 4 and 7) the percentage of drug resistance in Sardinian and foreign populations was similar (14% vs. 16%); however, among the immigrants there was a strain resistant to rifampicin and two MDR strains that were absent in the natives.

Data collected allowed us to find with a good approximation the phylogenetic relations among the strains isolated from natives and immigrants and with the best-known phylogenetic clusters. Regarding the strains isolated from immigrants, most of them find their place in groups related to the origin of the patients. Among them there were four strains (1, 3, 4, and 36) belonging to the Beijing group. All Sardinian strains were very similar and found their right place in a group not described in literature.

Table 5 – Variable number tandem repeat-mycobacterial interspersed repetitive units (VNTR-MIRU) genotyping of sardinians' clinical isolates.

ID	Town	VNTR-MIRU loci														
		424	577	580	802	960	1,644	1,955	2,163	2,165	2,401	2,996	3,192	3,690	4,052	4,156
1SS	Sassari	4	5	3	4	3	2	5	4	5	4	8	7	5	8	3
2SS	Sassari	4	5	4	3	3	2	1	2	5	3	6	2	1	3	3
3SS	Sassari	3	4	3	2	4	2	3	5	0	4	6	3	2	6	4
4SS	Sassari	0	4	3	4	0	2	3	3	3	4	6	0	0	5	4
5SS	Sassari	0	5	0	2	3	0	0	5	3	3	6	3	2	4	3
6SS	Sassari	0	0	3	0	4	2	0	0	0	2	5	0	1	0	3
7SS	Sassari	2	0	3	5	3	2	2	4	2	3	6	3	5	5	2
8SS	Sassari	2	5	3	5	3	2	2	5	4	3	6	3	2	5	3
9SS	Sassari	2	0	3	0	1	2	2	3	4	0	3	0	2	6	0
10SS	Sassari	3	3	3	4	4	2	3	0	0	2	6	0	4	4	2
11SS	Sassari	2	5	3	1	4	1	3	2	0	3	5	0	4	6	2
12SS	Sassari	2	5	0	4	3	2	2	2	0	3	6	0	6	5	0
13SS	Sassari	5	5	3	3	3	3	1	0	5	3	6	2	1	6	3
14SS	Sassari	0	0	4	6	3	2	0	0	0	3	5	0	2	0	3
15NU	Nuoro	0	0	3	0	4	3	0	0	0	2	5	0	1	0	4
16NU	Nuoro	3	5	3	1	3	3	4	4	4	3	6	2	4	3	3
17NU	Nuoro	2	5	3	2	3	5	3	2	2	2	6	3	1	6	4
18NU	Nuoro	0	0	3	0	4	2	0	0	0	5	6	0	2	0	4
19NU	Nuoro	0	0	3	0	5	2	0	0	0	5	6	0	2	0	4
20NU	Nuoro	3	5	3	2	3	2	3	2	2	2	6	3	1	7	3
21NU	Nuoro	4	5	0	2	2	2	0	0	3	2	6	0	4	6	0
22NU	Nuoro	0	0	3	0	5	2	0	0	0	2	6	0	1	0	3
23OT	Tempio	3	5	3	1	3	2	2	4	0	3	6	3	7	4	3
24OT	Olbia	4	4	3	4	3	1	3	7	4	4	6	3	5	3	2
25OR	Oristano	0	4	3	5	4	2	2	6	4	0	6	3	2	4	2

ID = identification.

Table 6 – Allelic diversity index (D) for loci of sardinians' strains.

MIRU	Allelic frequencies								*D*
	1	2	3	4	5	6	7	8	
424	0	6	5	4	1	0	0	0	*D* = 0.73
577	0	0	1	4	12	0	0	0	*D* = 0.63
580	0	0	20	2	0	0	0	0	*D* = 0.31
802	3	5	2	5	3	1	0	0	*D* = 0.82
960	1	1	13	7	2	0	0	0	*D* = 0.62
1644	2	18	3	0	1	0	0	0	*D* = 0.43
1955	2	6	7	1	1	0	0	0	*D* = 0.74
2163	0	5	2	4	3	1	1	0	*D* = 0.77
2165	0	3	3	5	3	0	0	0	*D* = 0.71
2401	0	8	10	4	2	0	0	0	*D* = 0.69
2996	0	0	1	0	4	19	0	1	*D* = 0.37
3192	0	2	9	0	0	0	1	0	*D* = 0.61
3690	1	8	0	4	3	1	1	0	*D* = 0.78
4052	0	0	3	4	4	6	1	0	*D* = 0.81
4156	3	5	11	6	0	0	0	0	*D* = 068
									Average = 0.65

MIRU = mycobacterial interspersed repetitive units.

Discussion

The migration wave from low-income and middle-income countries towards industrialized ones increased by 17% from 1990 to 2005; it has been estimated that foreign-born individuals legally living in developed countries are more than 190 million. Migration goes with matters of public health in host countries, since there is a possibility that immigrants could be transmitting infectious diseases to the native population. TB is one of the diseases that has shown an increase in incidence in industrialized countries as a result of migration. In particular, people coming from countries in which this disease is endemic are at high risk of developing TB from reactivation of a latent infection acquired in their countries

Table 7 – Antimycobacterial susceptibility pattern of sardinians' strains.

ID	Town	INH	RIF
1SS	Sassari	R	S
2SS	Sassari	R	S
3SS	Sassari	S	S
4SS	Sassari	S	S
5SS	Sassari	S	S
6SS	Sassari	S	S
7SS	Sassari	S	S
8SS	Sassari	S	S
9SS	Sassari	S	S
10SS	Sassari	S	S
11SS	Sassari	S	S
12SS	Sassari	S	S
13SS	Sassari	S	S
14SS	Sassari	S	S
15NU	Nuoro	S	S
16NU	Nuoro	S	S
17NU	Nuoro	S	S
18NU	Nuoro	S	S
19NU	Nuoro	R	S
20NU	Nuoro	S	S
21NU	Nuoro	S	S
22NU	Nuoro	S	S
23OT	Tempio	S	S
24OT	Olbia	S	S
25OR	Oristano	R	S

ID = identification; INH = isoniazid; R = resistant; RIF = rifampin; S = susceptible.

of origin. Italy is one of the 24 European countries with a low incidence of TB (7/100,000 inhabitants), but in the last few years there has been a rise in the disease, especially in the elderly, in human immunodeficiency virus-positive patients, and in immigrants from high-incidence countries [2,3].

Molecular epidemiological studies have allowed studies to reach very important results about the understanding of the transmission dynamics of the disease, providing great help with the development of intervention strategies for control [5,10].

Genotyping of *M. tuberculosis* isolates allows the finding of epidemiological links among TB patients, to detect unsuspected outbreaks, to distinguish exogenous reinfection from endogenous reactivation in relapse cases, and to acquire information about the pathophysiology of the strains [9]. Besides, molecular epidemiological studies allow the evaluation of the impact that subpopulations have on the transmission of the infection in a community [5,10].

In this work two groups of clinical isolates of *M. tuberculosis* were genotyped with the variable number tandem repeat (VNTR)-MIRU technique. The first group was composed of 36 isolates of immigrant patients of different ethnic groups living in Sardinia, the second one of 25 isolates of Sardinian patients coming from different districts of the island.

Regarding the immigrants, the results obtained with the VNTR-MIRU technique pointed out that the 36 isolates analyzed were different from each other. This diversity emerged not only among strains isolated from foreign-born people coming from different continents (Africa, Asia, and East Europe), but also in people belonging to the same ethnic group. The explanation may be found first of all in the high genetic variability of *M. tuberculosis* strains, widely described in literature and in surveys in which new accurate genotyping techniques have been studied [10]. Secondly, the clinical isolates had been collected over a 10-year period; therefore, it is possible that even if all the immigrants tested were in Sardinia, they could not have had any contact with each other. Moreover they might have come to Italy with a latent tubercular infection and developed the active disease as a consequence of the poor living conditions after their arrival into the country. According to this possibility, it is even more improbable that they could have had any contact, since the places they came from were very far away from one other. *M. tuberculosis* strains' diversity pointed out in each ethnic group may be explained in the same way.

A measure for this variability was given by the whole of the allelic diversity indexes *D* for each locus; the average $D = 0.75$ pointed out a high variability inside the group. Apart from locus 580, for which $D = 0.44$, a medium–low value, all *D* values were included in a medium–high range of 62–78.

The genotyping through the VNTR-MIRU technique of the 25 clinical isolates of the Sardinians showed that all the strains were different from each other and that there were no differences related to the place of origin of the individuals.

The average of the allelic diversity index for each locus, $D = 0.65$, pointed out a medium–high genetic variability. The allelic diversity was lower than that of the immigrants and yet it was still very high, considering that the isolates came from a narrower geographical area. The explanation may be found also in this case first of all in the high genetic variability of *M. tuberculosis* strains and secondly, the active disease could be due to the reactivation of a latent tubercular infection caused by a strain acquired in the past, the transmission of which did not necessarily occur in Sardinia or involve native individuals.

Allelic diversity indexes show that loci 580, loci 2996, and loci 1644 had a medium–low genetic variability. Uniformity in locus 580 was found also in the group of the immigrants and in both cases was due to the fact that most of the strains had the same number of repeated copies (three). Since there have been no data in literature and considering that the taxonomic positions of the strains were very near, it is possible that it was a feature of the strains analyzed, due to a common phylogenetic origin.

Concerning MIRU 2996, most strains of the native population, and nearly all the Romains among the immigrants had the same allelic frequency (six), while for MIRU 1644 this happened only for Sardinians' strains (two). Also in these cases, an evaluation in light of taxonomical data led to comments similar to those for MIRU 580.

A comparison between the two groups pointed out that there were no identical strains between the immigrants and the Sardinians. This may indicate that there was no epidemiological link between the two populations and that none of them had influenced the transmission of TB in the other one. Actually, it is necessary to point out that the number of clinical isolates was small in light of the high genetic diversity found; therefore, caution is required in the inference to the whole population.

The execution of the first-line antimycobacterial susceptibility test showed a similar situation for the two populations about the percentage of drug resistance. However, only in the immigrant population were there strains resistant to rifampicin and MDR strains. This result was in line with other studies in literature, which have described immigrants as a population with a high incidence of TB caused by strains with multiple resistances and MDR, mainly because of the lack of adequate therapeutic protocol in their countries of origin that favor the appearance of resistant strains. It is important to report that there were no strains with multiple resistances or MDR strains among the Chinese, which from a taxonomic perspective belonged to the Beijing group, known for a significant presence of these kind of strains.

Conclusions

The results of genotyping, which should be considered preliminary in light of the remarks above, have allowed us to get a picture of the number and the kind of *M. tuberculosis* strains present in the immigrant population and to acquire information about the strains of Sardinian patients, about which there was none. Moreover, the analysis showed that there is no evidence of TB transmission among immigrants and natives, thus confirming that the presence of immigrants is not a risk per se for the whole community.

However, in order to reach a better understanding of the dynamics of transmission of TB in this land, it is necessary to continue genotyping studies, collecting at the same time a higher number of information through routine public health investigations; only in this way will it be possible to develop public health intervention strategies aimed at individuals and populations at risk in order to gain an effective control of TB in the community.

Conflicts of interest

The authors declare that they have no competing interests.

REFERENCES

[1] WHO, Global Tuberculosis Report 2015, World Health Organization, Geneva, 2013.
[2] M. Conversano, Update on the epidemiology of tuberculosis in Italy, J. Rheumatol. Suppl. 91 (2014) 4–10.
[3] Italian National Centre of Epidemiology, Surveillance and Health Promotion, 2015. <www.epicentro.iss.it>.
[4] Italian National Centre of Statistics, 2015. <www.demoistat.it>.
[5] Statistics on Immigration, 2015. <www.ec.europa.eu>
[6] M.V. Burgos, A.S. Pym, Molecular epidemiology of tuberculosis, Eur. Respir. J. Suppl. 36 (2002) 54s–65s.
[7] A. Odone, M. Riccò, M. Morandi, et al, Epidemiology of tuberculosis in a low incidence Italian region with high immigration rates: differences between not Italy-born and Italy-born TB cases, BMC Public Health 11 (2011) 376.
[8] F. Franzetti, L. Codecasa, A. Matteelli, et al, Genotyping analysis of tuberculosis transmission among immigrant resident in Italy, Clin. Microbiol. Infect. 16 (2010) 1149–1154.
[9] A. Sandgren, M.S. Schepisi, G. Sotgiu, et al, Tuberculosis transmission between foreign- and native-born populations in the EU/EEA: a systematic review, Eur. Respir. J. 43 (2014) 1159–1171.
[10] M. an der Werf, J.P. Zellweger, Impact of migration on tuberculosis epidemiology and control in the EU/EEA, Euro Surveill. 21 (2016).
[11] P.F. Barnes, M.D. Cave, Molecular epidemiology of tuberculosis, N. Engl. J. Med. 349 (2003) 1149–1156.
[12] Protocol, Isolation of high molecular weight genomic DNA from Mycobacteria (CTAB procedure), 2010. <www.rivm.nl>.
[13] S. Masala, P. Molicotti, A. Bua, et al, Molecular characterization of Sardinian *Mycobacterium tuberculosis* isolates by IS6110 restriction fragment length polymorphism, MIRU-VNTR and rep-PCR, New Microbiol. 33 (2010) 155–162.
[14] P. Supply, C. Allix, S. Lesjean, et al, Proposal for standardization of optimized mycobacterial interspersed repetitive-unit-variable-number repeat typing of *Mycobacterium tuberculosis*, J. Clin. Microbiol. 44 (2006) 4498–4510.
[15] P. Supply, Multilocus variable number tandem repeat genotyping of Mycobacterium tuberculosis, Technical Guide, INSERM U629 Institut de Biologie/Institut Pasteur de Lille, May 2005.
[16] J. Palomino, M. Anandi, M. Camacho, et al, Resazurin microtiter assay plate: simple and inexpensive method for detection of drug resistance in *Mycobacterium tuberculosis*, Antimicrob. Agents Chemother. 46 (2002) 2720–2722.